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DATE: June 11, 2008

TO: Examiner Yong D. Pak
Group Art Unit 1652

PHONE #: 571-272-0935
FAX #: 571-273-8300

Application No.: 10/090,965 OUR REF.: 5005.01US02
Applicant: Srienc et al.
Due Date: June 17, 2008

FROM: Curtis B. Herbert, Ph.D.
PHONE #: 612-605-1038

Attached is the following for filing in the above-identified application.

(1) Appeal Brief Transmittal;
(2) Appeal Brief; and
(3) Carlson et al. *App. Env. Microbiol.*

Respectfully submitted,

Curtis B. Herbert, Ph.D.
Registration No. 45,443

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this paper is being transmitted by facsimile to the U.S. Patent and Trademark Office, Fax No. 571-273-8300 on the date shown below.

June 11, 2008
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Attorney Docket No. 5005.01US02

APPEAL BRIEF TRANSMITTAL

In re the application of:

	Srienc et al.	Confirmation No.: 6415
Application No.:	10/090,965	Examiner: Pak, Y.
Filed:	March 4, 2002	Group Art Unit: 1652
For:	PRODUCTION OF POLYHYDROXYALKANOATES	

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith, in triplicate, is the Appeal Brief in the above-identified application, with respect to the Notice of Appeal filed on April 17, 2008.

[X] Applicant(s) is/are entitled to small entity status in accordance with 37 CFR 1.27.

[X] The Commissioner is authorized to charge Deposit Account No. 50-3863 in the amount of [ ] $505.00 (large entity) [X] $255.00 (small entity) to cover the filing fee.

Respectfully submitted,

Curtis B. Herbert, Ph.D., Esq.
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Please grant any extension of time necessary for entry; charge any fee due to Deposit Account No. 50-3863.

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June 11, 2008
Date

Curtis B. Herbert, Ph.D., Esq.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Attorney Docket No.: 5005.01US02

Srienc et al. Confirmation No.: 6415

Application No.: 10/090,965 Examiner: Pak, Y.

Filed: March 4, 2002 Group Art Unit: 1652

For: PRODUCTION OF POLYHYDROXYALKANOATES

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

BRIEF FOR APPELLANT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Attorney Docket No.: 5005.01US02

Srienc et al. Confirmation No.: 6415

Application No.: 10/090,965 Examiner: Pak, Y.

Filed: March 4, 2002 Group Art Unit: 1652

For: PRODUCTION OF POLYHYDROXYALKANOATES

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES
APPEAL BRIEF

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

This is an appeal of the twice rejection of claims 1-13. The claims have been rejected in the office action mailed October 19, 2007 and in previous office actions, including March 22, 2007. A Notice of Appeal was filed April 17, 2008, less than two months from the present date. This Appeal Brief is thus timely filed.

Please grant any extension of time necessary for entry; charge any fee due to Deposit Account No. 50-3863.

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June 11, 2008
Date

Curtis B. Herbert, Ph.D., Esq.

06/11/2008 HMARZI1 00000057 503863 10090965

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REAL PARTY IN INTEREST

Dr. Friedrich Srienc, an inventor of the application, has acquired the entire right, title and interest in and to the invention, the application, and any and all patents to be obtained therefore, as per the Assignment, recorded at Reel 020815, Frame 0362.

RELATED APPEALS AND INTERFERENCES

The assignee of the present application has no other applications presently on appeal. Since no other decided appeals are related in any relevant way to the present appeal, no earlier opinions are attached in the Related Appeals Appendix.

STATUS OF CLAIMS

Claims 1-13 are pending, and all of the pending claims stand rejected. Claims 14-94 have been canceled. The pending claims are listed in the Appendix 1. All pending claims are being appealed.

STATUS OF AMENDMENTS

All amendments have been entered.

SUMMARY OF CLAIMED SUBJECT MATTER

The present invention is directed to a method for the production of a polyhydroxyalkanoate (PHA). Transgenic yeast cells are cultured under anaerobic conditions to cause production of the PHA. Application, page 4 lines 3-4. The yeast cells have heterologous nucleic acids that encode PHA polymerase as well as those selected from the group of acetoacetyl-CoA reductase and β-

ketothiolase. Application, page 4 lines 3-9. The claimed anaerobic conditions must provide an average yield of PHA in the culture is at least about 1.5% of dry cell weight. Application, e.g., Example 10 starting at page 90, see sentence bridging pages 90-91.

The claimed polyhydroxyalkanoates (PHAs) are polyester polymers of hydroxyalkanoate monomers conforming to the general structure illustrated in FIG. 1 of the Application. Formation of PHA in the yeast occurs by way of polymerization of the monomers and is catalyzed by a heterologous PHA polymerase. Application, page 3 lines 16-20. PHA is a term that includes various alkanoates, including poly-3-hydroxybutyrate (PHB). Application, page 8 lines 30-31. Figure 2 depicts one metabolic pathway interconnected with other metabolic pathways in yeast, specifically, a biochemical scheme for making the claimed PHA, with the three labeled boxes indicating enzymes of claim 1: β-ketothiolase (top box), acetoacetyl-CoA reductase (middle box) and PHA polymerase (bottom box labeled "synthase").

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. The rejection of claims 1-13 as unpatentable under 35 U.S.C. §103(a) as being unpatentable over Madison et al., Johnston et al., Clemente et al., and Linde et al..

ARGUMENT

GROUPING OF CLAIMS

Group 1. Claims 1-13 are directed to a method for the production of a polyhydroxyalkanoate.

LEGAL AUTHORITY

A. The Examiner Bears The Burden Of Demonstrating Obviousness.

The patent office has the burden of persuasion in showing that the Applicants are not entitled to a patent. "[T]he conclusion of obviousness vel non is based on the preponderance of evidence and argument in the record." In re Oetiker, 24 USPQ2d 1443, 1445 (Fed. Cir. 1992). The patent office has the ultimate burden of persuasion in establishing that an applicant is not entitled to a patent. Id. at 1447, concurring opinion of Judge Plager. **"The only determinative issue is whether the record as a whole supports the legal conclusion that the invention would have been obvious."** Id. "In rejecting claims under 35 U.S.C. §103, the examiner bears the initial burden of presenting a prima facie case of obviousness." In re Rijckaert, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). If the Examiner fails to establish a prima facie case of obviousness, the obviousness rejection must be withdrawn as a matter of law. In re Ochiai, 37 USPQ at 1131 ("When the references cited by the examiner fail to establish a prima facie case of obviousness, the rejection is improper and will be overturned"). "If examination at the initial stage does not produce prima facie case of unpatentability, then without more the applicant is entitled to grant of the patent." In re Oeitiker, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992).

"Only if that burden is met, does the burden of coming forward with evidence or argument shift to the applicant." In re Rijckaert, 28 USPQ2d at 1956. "After evidence or argument is submitted by the applicant in response to an obviousness rejection, 'patentability is determined on the totality of the record, **by a preponderance of the evidence** with due consideration to persuasiveness of the argument.'" In re Chu, 36 USPQ2d 1089, 1094 (Fed. Cir. 1995)(quoting In re Oetiker, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992), emphasis added).

B. Results not predicted by the prior art are evidence of patentability.

The Supreme Court has held that "The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results." KSR Intern. Co. v. Teleflex Inc., 127 S.Ct. 1727, 1739 (2007). Specifically, "a court must ask whether the improvement is more than the predictable use of prior art elements according to their

established functions." Id. at 1731. "Often, it will be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person of ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue." Id. at 1740. The Court noted that "it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." Id. at 1731. This is so because "inventions [in most, if not all, instances] rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known." Id.

C. The References Must Provide A Reasonable Expectation Of Success

While a reference is prior art for all that it teaches, references along with the knowledge of a person of ordinary skill in the art must be enabling to place the invention in the hands of the public. In re Paulsen, 31 USPQ2d 1671, 1675 (Fed. Cir. 1994). See also In re Donohue, 226 USPQ 619, 621 (Fed. Cir. 1985). "The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood success, viewed in light of the prior art." Micro Chemical Inc. v. Great Plains Chemical Co., 41 USPQ2d 1238, 1245 (Fed. Cir. 1997)(quoting In Re Dow Chemical Co., 5 USPQ2d 1529, 1531 (Fed. Cir. 1988)).

Obviousness does not require absolute predictability, but at least some degree of predictability is required. In the case Amgen v. Chugai, 927 F.2d 1200,(C.A.F.C. 1991) the court upheld a finding of nonobviousness since there would have been "**no more than a fifty percent chance of success**" in cloning the disputed EPO gene using prior art methods. 927 F.2d 1200,

1208, emphasis added. The court further pointed to the well established principle that "Both the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure." 927 F.2d 1200, 1208.

D. Prior art that teaches away from the invention is evidence of patentability, including prior art leading artisans away from the path taken by the applicant.

"There is no suggestion to combine [references] if a reference teaches away from its combination with another source." *Tec Air Inc. v. Denso Manufacturing Michigan Inc.*, 52 USPQ2d 1294, 1298 (Fed. Cir. 1999). "A reference will teach away if it suggests that a line of development flowing from the reference's disclosure is unlikely to be productive of the result sought by the applicant." *Winner International Royalty Corp. v. Wang*, 53 USPQ2d 1580, 1587 (Fed. Cir. 2000)(quoting *In re Gurley*, 31 USPQ2d 1130, 1131 (Fed. Cir. 1994)). "A reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant." *In re Gurley*, 31 USPQ2d 1130, 1131 (Fed. Cir. 1994).

E. The Patent Office Must Provide a Rationale Based Squarely on the Prior Art to Reject an Invention.

The Supreme Count has confirmed the warnings in Graham against hindsight analysis by noting that a "factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon *ex post* reasoning." KSR Int'l Co., 127 S.Ct. 1727, at 1742.

The importance of the principle that the prior art itself must suggest the motivation to modify the teachings of a reference was eloquently stated in In re Rouffet, 47 USPQ2d 1453, 1458 (Fed. Cir. 1998)(emphasis added):

> The Board did not, however, explain what specific understanding or technical principle within the knowledge of one of ordinary skill in the art would have suggested the combination. **Instead the board merely invoked the high level of skill in the field of the art. If such a rote invocation could suffice to supply a motivation to combine, the more sophisticated scientific fields would rarely, if ever, experience a patentable technical advance.** Instead, in complex scientific fields, the Board could routinely identify the prior art elements in an application, invoke the lofty level of skill, and rest its case for rejection. **To counter this potential weakness in the obviousness construct, the suggestion to combine requirement stands as a critical safeguard against hindsight analysis and rote application of the legal test for obviousness.**

ANALYSIS

The claims stand rejected under 35 U.S.C. §103(a) in light of Madison et al. (Micro. Mol. Biol. Rev., 1999; 63(1):21-53), Clemente et al. (U.S. Pat. No. 5,849,894), Linde et al. (J. Bacteriology, Dec. 1999; 181(24):7409-7413), and Johnston et al. (Microbiol. Reviews, 1987; 51(4):458-476). The rejection is made out in the office action of October 19, 2007 (the Office Action).

As detailed below, this rejection is traversed on the grounds that the prior art did not predict the success of the claimed invention and taught against the invention because the artisan would have been lead away from what is claimed by the prior art. Therefore it is respectfully argued that the Patent Office has not made out a prima facie case of obviousness. Further, Applicant's results point to previously unknown cellular behaviors, which is a surprising result that provides secondary evidence of nonobviousness even if the Patent Office maintains that a case of prima facie obviousness has been maintained. These arguments are made in the context of an unpredictable art such that the claimed invention is more than a predictable result of prior art elements.

The argument for obviousness in the Office Action

Claims 1-13 stand rejected for obviousness in light of Madison et al., Johnston et al., Clemente et al., and Linde et al. The Office Action (at page 3) generally takes the position that Madison et al. suggests, at page 44 of Madison et al, improving polyhydroxyalkanoate (PHA) yields in yeast by increasing the activity of beta-ketothiolase and acetoacetyl-CoA reductase. The Office Action explains its position that expressing enzymes transgenically is well known (pages 3-4, referring to Clemente et al. and other references). Then the Office Action looks to Linde et al. and other art to support the idea that yeast are known to grow effectively in aerobic and anaerobic conditions such that one of ordinary skill in the art "would have recognized to use transgenic [yeast] under anaerobic or aerobic conditions, permitting flexibility in culture conditions" (Office Action page 4 and 5).

The Office Action points to a reasonable expectation of success since Madison et al. teach that an increase in activity of β-ketothiolase and an acetoacetyl-CoA reductase in yeast transformed with PHSSCL or PHAMCL will increase the yield of PHA, the expression of multiple genes in yeast in known by Clemente et al. and Johnston et al., and Linde et al. teaches flexibility of expressing genes aerobically and anaerobically (Office Action page 5).

In general, the problem with this rationale is that it does not consider that PHA is a metabolic product and instead looks to a rationale based on recombinant DNA expression to make out a rejection. A quantity of PHA is claimed as produced by culture conditions that are contrary to conventional wisdom and would not have been reasonably expected to proceed.

The claimed invention is a metabolic product; The prior art leads artisans away from the path taken by Applicant.

The claims are directed to production of PHA. The claimed PHA is a metabolic product and not a protein expressed by familiar techniques of recombinant DNA expression. The claimed

enzymes are recombinantly expressed and must consume other cell resources to produce the claimed PHA metabolic product. Application, e.g., page 59 lines 18-31.

The breakdown of energy sources to release energy (termed catabolism) is a key metabolic activity that ultimately requires an electron acceptor or "sink". The progress of electrons through a metabolic pathway can be described in terms of a "reducing equivalent" which is a term that refers to a chemical that transfers the equivalent of one electron in an oxidation-reduction (redox) reaction. Examples of reducing equivalents are a hydrogen atom (consisting of a proton and an electron) or a hydride ion (H^-) which carries two electrons (for example in reactions involving NADH). In aerobic conditions, yeast grow ("oxidative growth") and NADH is made by catabolism. The NADH is transferred to the electron transport chain where O_2 is reduced to H_2O in a process that generates adenosine triphosphate (ATP). Application, e.g., paragraph bridging pages 20-21.

A lack of redox balance in the cell leads to cell death; in other words, there must not be too many or too few H^- ions. Anaerobic conditions pose a redox challenge because the electron transport system is not available to accept electrons (there is effectively no oxygen). Instead, the reducing equivalents are transferred to a metabolite that acts as an electron "sink". The reduced metabolites are then typically exported from the cell. The reduction of acetaldehyde to ethanol is a common example. The yeast effectively retool their metabolic machinery to produce ethanol in anaerobic conditions to offload excess H^- so as to survive. Application, e.g., page 20 line 24 to page 21 line 12.

NADP is a cofactor that binds H^- ions to make NADPH. NADPH is involved in metabolic pathways that are not directed to catabolism or offloading of H^- ions, although the availability of many factors in a cell inevitably provides some linkages between its metabolic pathways. NADPH is used in anabolic reactions (such as lipid and nucleic acid synthesis). Application, e.g., page 105 lines 9-17. NADPH is formed primarily through operation of the pentose phosphate cycle

Application, e.g., page 21 lines 5-12. The prior art does not indicate how much of the cell's metabolism is directed into this cycle, or how to reliably predict how this metabolism will be directed.

Significantly, the ratio of NADPH to NADP is key for PHA production, with a high ratio favoring PHA production (See "The availability of reducing equivalents in the form of NADPH is therefore considered to be the driving force for P(3HB) [a type of PHA] formation." Madison et al., page 27, last sentence of first full paragraph.)

In the case of PHA production, it was quite problematic to contemplate successfully producing a metabolite that depends on NADPH/NADP ratios under conditions that are known to re-orient the cells to offload excess H ions as ethanol, i.e., in anaerobic culture. In this case, when the cell's metabolic machinery is dedicated to making ethanol for survival, trying to intervene in that path to divert resources away from life-saving H^- consumption to PHA production was counterintuitive. In effect, the cell has retooled itself to make ethanol and has created a pathway that demands H^- ions. The ethanol pathway's consumption of H^- ions can fairly be expected to drive NADPH/NADP ratios down so that PHA production is minimized.

And, as another layer of complication, yeast cells must also maintain a delicate balance between NADPH and NADH production and consumption to maintain a redox balance. Application, e.g., page 21 lines 17-20. Perturbations in the concentrations of the NADPH/NADP ratio, such as those caused by the introduction of a catabolic or anabolic pathway, can often lead to an unfavorable redox balance. Application, e.g., page 21 lines 15-17. While many cells have a transhydrogenase system that permits interconversion between NADPH and NADH so as to compensate between its need for one or the other, it is conventionally believed that yeast do not have this capability. See Application, "Transhydrogenase Systems" on page 21.

But the results in the Application unexpectedly suggest that **PHA can serve as a sink** for NADH during **anaerobic metabolism**. Application page 11 lines 21-22. Conventional wisdom

provides no prediction of this capability and has no ready explanation. Evidently, however, PHA can substitute for a normal fermentation product and accumulate as a fermentation product substitute within the cell. This result implies at the same time that there is a mechanism to convert the excess NADH into NADPH to enable PHA synthesis, despite the fact that there is no conventionally known transhydrogenase system for their interconversion. Alternately this could indicate that NADH can be directly used for intracellular PHA formation. For all of these reasons, an artisan would not have reasonably expected anaerobic fermentation to be a successful process for PHA production.

In fact, PHA is conventionally considered to be an aerobic storage material in cells. Application page 15 line 8. And β-oxidation of fatty acids, which is generally a step needed for PHA production, is an aerobic process, such that removing oxygen from the system could fairly be expected to reduced the precursor pool and thereby inhibit PHA production. Application page 15 lines 9-11. Making aerobic storage materials in anaerobic conditions is against conventional wisdom.

These considerations lead to specific points as follows.

Production of PHA as claimed by engineering of metabolic pathways is an unpredictable art such that there would be no reasonable success for making the claimed invention; the prior art teaches away from what is claimed because artisans would not expect PHAs as claimed to be produced anaerobically.

The literature explicitly states that these are unpredictable arts such that there would be no reasonable expectation of success for making the claimed invention. Madison et al. state that "Taken together, these molecular genetic data provide a glimpse of the complexity of PHA metabolism. Since PHA formation **is dependent on the fluxes in central metabolic pathways and the levels of precursors**, a detailed knowledge of the molecular physiology of PHA metabolism is ***critical*** for successful implementation of transgenic PHA producers. ***Unlike the***

production of heterologous proteins, which relies mostly on sufficient gene expression, recombinant PHA production involves coordinated expression of heterologous enzymes over a prolonged period and with a concomitant redirection of the metabolism of the host. As a consequence of the metabolic changes introduced by expressing the *pha* and *phb* genes, the cell will induce its own responses, *which are not necessarily favorable for PHA production.* It is therefore ***critical*** to understand how bacteria normally **regulate PHA formation** and how undesired responses from a recombinant host can be prevented. ***Only then*** can recombinant processes be successfully developed and lead to what are expected to be the most efficient PHA production processes." Madison et al, at page 35 under "Conclusions", emphases added.

Madison et al. thus explains that merely expressing certain genes does not provide even a *punctilio* of predictability for PHA production. It is one thing to have a few heterologous proteins expressed but quite another to have any meaningful amount of PHA produced, e.g., the claimed at 1.5% of dry cell weight. There is no contradictory evidence in the record on this point even though the Patent Office's rationale is in direct opposition to Madison et al.

As explained above, PHA is conventionally considered to be an aerobic storage material in cells. Application page 15 line 8. Making aerobic storage materials in anaerobic conditions is manifestly against conventional wisdom. The prior art must therefore lead the artisan towards use aerobic growth conditions and away from the claimed anaerobic conditions. There is no contradictory evidence in the record on this point.

As explained above in detail, in the case of PHA production, it was contrary to conventional wisdom to contemplate successfully producing a metabolite that depends on NADPH/NADP ratios under conditions that are well known to re-orient the cells to offload excess H^+ ions as ethanol, i.e., in anaerobic culture. In this case, when the cell's metabolic machinery is dedicated to making ethanol for survival, trying to intervene in that path to divert resources away

from life-saving H^- consumption to PHA production was counterintuitive. The prior art would lead the artisan to use aerobic growth conditions and away from the claimed anaerobic conditions. There is no contradictory evidence in the record on this point.

Applicants discovered that PHA can evidently substitute for a normal fermentation product and accumulate as a fermentation product substitute within the cell. PHA can apparently serve as a sink for NADH during anaerobic metabolism. Conventional wisdom provides no prediction of this capability and has no ready explanation. Without this capability, it could not be expected that the claimed method would succeed. Since a new scientific theory was required for the successful production of PHA in the conditions and quantities claimed, the claimed production of PHA is an unexpected and surprising result that is secondary evidence of nonobviousness. There is no contradictory evidence in the record on this point.

The teachings of Linde et al are irrelevant to predicting success of production of PHA in anaerobic cultures

The Office Action relies on Linde et al. as teaching the flexibility of genes aerobically or anaerobically (page 5). The teachings of Linde et al. are irrelevant, however, to predicting success of production of PHA in anaerobic cultures because Linde et al. is directed to the production of heterologous proteins, but what is claimed involves redirection of the metabolism of the host - not merely the expression of particular genes. As explained in the passage of Madison et al. quoted above, even if Linde et al. were assumed to predict the success of expression of certain proteins, **this would not be enough to predict success** because it does not speak to the critical aspects of the cell's metabolic pathways.

The Office Action's response to Applicant's arguments.

The Office Action made some responses to Applicant's arguments starting at page 5 therein. The Office Action argued that Linde et al. teaches that yeasts are unique in exhibiting

fast growth in the presence or complete absence of oxygen such that there would be a reason to use transgenic yeast in anaerobic culture so as to permit flexibility and thereby improve cost effectiveness of producing PHA. Respectfully, however, this argument does not speak to Applicant's point about Linde et al., which is that Linde et al. is directed to the production of heterologous proteins, but what is claimed involves redirection of the metabolism of the host - not merely the expression of particular genes, as discussed in detail herein. The rationale that artisans would do what the Applicant has done to save money is, respectfully, mere hindsight taken from the Application that teaches what is claimed is possible.

The Office Action argued that an artisan would, in fact, expect successful anaerobic production of PHAs (page 5 Office Action), and points to Lee et al. (Waste Management 19 (1999) at page 134 therein. Respectfully, Lee et al. stands for the proposition that what is apparently anaerobic synthesis of PHA can take place in **consortia** of **bacteria** such as in the activated sludge of wastewater plants. To conclude from this that **yeast** can make PHA anaerobically is not a fair inference since the poorly understood activity of bacteria cannot be fairly attributed to yeast, which are different organisms, and since it is necessarily unknowable if a not-understood mechanism would be operable in a much different setting, namely, yeast cultures. In fact, anaerobic synthesis has not been studied in pure cultures and therefore its mechanism and regulation is not well understood.

Objective evidence in support of these facts is provided by the attached peer-reviewed publication entitled Kinetic Studies and Biochemical Pathway Analysis of Anaerobic [PHA] Synthesis in *Escherichia coli*:

> "PHA production under conditions of oxygen stress and in the absence of oxygen has been reported for organisms that natively accumulate PHA (3, 4, 45). Anaerobic PHA production has been studied in undefined bacterial consortia found in a wastewater treatment process known as enhanced

biological phosphorous removal (for recent reviews, see references 31 and 46). These studies examined a still unclear relationship between a PHA-accumulating bacterial consortium and the removal of phosphorous compounds from wastewater streams (37)." Carlson et al., *App. Env. Microbiol.*, Feb 2005, 713-720 (2005), at page 713, third paragraph.

Significantly, the last paragraph of the Discussion section of Carlson et al., see *App. Env. Microbiol.*, Feb 2005, 713-720 (2005), points out that yeast lacks a transhydrogenase system that would convert NADH into NADPH. Under anaerobic growth conditions, NADH accumulates in the cells but it is well-known that NADPH is needed for PHA synthesis. As already stated, the Applicants in the instant case have discovered that there is a mechanism to convert the excess NADH into NADPH to enable PHA synthesis, despite the fact that there is no conventionally known transhydrogenase system for their interconversion. This fact underscores the differences between the claimed yeast systems and the bacterial systems cited in the Office Action.

Respectfully, the Office Action's reliance on a poorly-understood bacterial consortium to predict yeast function underscores the weakness of the Patent Office's case; no artisan of ordinary competence would accept the assertion that such a system would have useful predictive value for the claimed yeast.

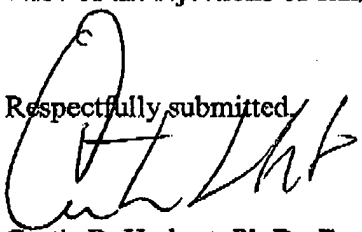
The Office Action suggests that Applicants provide no evidence that these are unpredictable arts, and requires explicit statements in the prior art that anaerobic PHA production is not expected or difficult. The Applicants response, however, make multiple specific point cites to publications that plainly state that PHA production is an unpredictable art. If PHA production is unpredictable, it is an error in logic to assume that anaerobic PHA production is predictable for anaerobic conditions. Evidence that PHA production is an unpredictable art is of-record and can not be ignored or dismissed.

Another point made in the Office Action is that absolute certainty is not required to combine the references (page 6). This point is acknowledged. The record, however, including Madison et al., **plainly states** that PHA metabolism is an unpredictable art. The record provides multiple unrebutted reasons why an artisan would not expect success, much less success in the claimed quantities. The record shows that an apparently new scientific discovery was required to understand why what is claimed is successful.

CONCLUSIONS

Applicants believe that the Patent Office has failed to meet its burden of persuasion with respect to unpatentability of any of the claims on the present record. Thus, Applicants Respectfully request the Appeals Board to reverse of the rejections of claims 1-13.

Respectfully submitted,



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CLAIMS APPENDIX

PENDING CLAIMS

1. A method for the production of a polyhydroxyalkanoate (PHA) comprising:

providing a transgenic yeast cell comprising a first nucleic acid fragment comprising a heterologous nucleotide sequence encoding a PHA polymerase and at least one second nucleic acid fragment comprising a heterologous nucleotide sequence selected from the group consisting of a heterologous nucleotide sequence encoding an acetoacetyl-CoA reductase and a heterologous nucleotide sequence encoding a β-ketothiolase;

culturing the transgenic yeast cell in a culture under anaerobic conditions to cause the production of PHA; and

isolating the PHA from the cell;

wherein an average yield of PHA in the culture is at least about 1.5% of dry cell weight of the culture.

2. The method of claim 1 wherein the first and second nucleic acid fragments constitute a single nucleic acid fragment.

3. The method of claim 2 wherein the single nucleic acid fragment comprises a divergent promoter operably linked to two of the heterologous nucleotide sequences.

4. The method of claim 1 wherein the yeast cell comprises a second nucleic acid fragment comprising a heterologous nucleotide sequence encoding an acetoacetyl-CoA reductase and a third nucleic acid fragment comprising a nucleotide sequence encoding a β-ketothiolase.

5. The method of claim 4 wherein at least two of the first, second and third nucleic acid fragments constitute a single nucleic acid fragment.

6. The method of claim 5 wherein the single nucleic acid fragment comprises a divergent promoter operably linked to two of the heterologous nucleotide sequences.

7. The method of claim 1 wherein at least one nucleic acid fragment is integrated into the genome of the yeast cell.

8. The method of claim 1 further comprising introducing at least one nucleic acid fragment into the yeast cell to yield the transgenic yeast cell.

9. The method of claim 1 wherein the yeast cell is a cell from the genus *Saccharomyces*.

10. The method of claim 1 wherein the yeast cell is an *S. cerevisiae* cell.

11. The method of claim 1 wherein the yeast cell is a cell from the genus *Kluyveromyces*.

12. The method of claim 1 wherein the PHA polymerase comprises a PHA_{SCL}.

13. The method of claim 1 wherein the PHA polymerase comprises a PHA_{MCL} polymerase.

14-94 (Cancelled)

EVIDENCE APPENDIX

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Kinetic Studies and Biochemical Pathway Analysis of Anaerobic Poly-(*R*)-3-Hydroxybutyric Acid Synthesis in *Escherichia coli*

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Poly-(*R*)-3-hydroxybutyric acid (PHB) was synthesized anaerobically in recombinant *Escherichia coli*. The host anaerobically accumulated PHB to more than 50% of its cell dry weight during cultivation in either growth or nongrowth medium. The maximum specific PHB production rate during growth-associated synthesis was approximately 2.3 ± 0.2 mmol of PHB/g of residual cell dry weight/h. The by-product secretion profiles differed significantly between the PHB-synthesizing strain and the control strain. PHB production decreased acetate accumulation for both growth and nongrowth-associated PHB synthesis. For instance under nongrowth cultivation, the PHB-synthesizing culture produced approximately 66% less acetate on a glucose yield basis as compared to a control culture. A theoretical biochemical network model was used to provide a rational basis to interpret the experimental results like the fermentation product secretion profiles and to study *E. coli* network capabilities under anaerobic conditions. For example, the maximum theoretical carbon yield for anaerobic PHB synthesis in *E. coli* is 0.8. The presented study is expected to be generally useful for analyzing, interpreting, and engineering cellular metabolisms.

Anaerobic cultivation is often used for producing commercial biochemicals like ethanol, lactate, succinate, and 1,3 propanediol (10, 14, 22). Anaerobic conditions also play critical roles in several environmental and ecological processes (46, 50). From a metabolic modeling perspective, anaerobic culturing conditions present an interesting system for studying cellular strategies for maintaining a redox balance. The absence of an external electron acceptor requires the transfer of reducing equivalents to metabolic intermediates which are usually easily quantifiable and provide a means of probing the intracellular workings of a microbe.

Poly-(*R*)-3-hydroxybutyric acid (PHB) belongs to a family of naturally occurring, biodegradable polyesters, known as polyhydroxyalkanoates (PHA) (for a recent review see reference 51). These materials act as reserve compounds for carbon, energy, and reducing equivalents and are of interest because their material properties make them a potential alternative to some petroleum-based thermoplastics. Recombinant *Escherichia coli* systems have been used extensively to study PHA production (for instance, see references 16, 24, 32, 33, 39, and 47). The effect of oxygen stress on recombinant *E. coli* cultures has been examined (52, 54); however, these studies all fed various amounts of oxygen and were not strictly anaerobic. PHB production in the absence of oxygen represents an interesting strategy for large-scale biopolymer production because anaerobic culturing typically permits simpler reactor design, control strategies, and operating conditions. For instance, since the low aqueous solubility of oxygen is not an issue, the culture is not subject to oxygen mass transfer limitations or steep oxygen gradients, which can cause experimental variation when there is nonideal reactor mixing. Anaerobic systems also have the added benefit of potentially coproducing other valuable by-products like ethanol, lactate, succinate, or hydrogen.

PHA production under conditions of oxygen stress and in the absence of oxygen has been reported for organisms that natively accumulate PHA (3, 4, 45). Anaerobic PHA production has been studied in undefined bacterial consortia found in a wastewater treatment process known as enhanced biological phosphorous removal (for recent reviews, see references 31 and 46). These studies examined a still unclear relationship between a PHA-accumulating bacterial consortium and the removal of phosphorous compounds from wastewater streams (37).

Elementary mode analysis is used here to study theoretical aspects of anaerobic PHA production. This method uses a field of mathematics known as convex analysis to identify all possible, unique, nondivisible pathways for a network (40–43). These pathways represent the simplest, steady-state flux patterns available to a system. Under steady-state conditions, the metabolic fluxes of an organism can be expressed as a nonnegative linear combination of elementary modes (12, 13). Elementary mode analysis has been used to study *E. coli* biochemical network properties (29, 41, 43, 49); however, few studies have examined the operation of foreign pathways engineered into a recombinant host (11). The network modeling approach provided a structured basis to interpret experimental results and defined explicitly the theoretical capabilities of the anaerobic *E. coli* metabolism. The presented study should therefore be generally useful for analyzing, interpreting, and engineering different aspects of cellular metabolisms.

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MATERIALS AND METHODS

Experimental system. *E. coli* DH5α (Invitrogen, Carlsbad, Calif.) was used in all experiments. Two different strains were created by transforming DH5α with

different plasmids. Plasmid pPT500 contains the native, three-gene *Ralstonia eutropha* PHB operon from pAeT41 (32) ligated into the pCR Blunt vector (Invitrogen). The operon is efficiently expressed in *E. coli* by the native *R. eutropha* operon promoter, which resembles the *E. coli* σ^{70} system (48). This *E. coli* strain is referred to as PHB(+). The other plasmid, pCR-KT, was constructed by ligating the *R. eutropha* β-ketothiolase gene, without a promoter, into the pCR Blunt vector. The control strain harboring this plasmid does not express any PHB genes and is referred to as PHB(−).

Culturing conditions. For growth studies, the strains were cultured in 2× YT medium:16-g/liter tryptone (Difco, Detroit, Mich.), 10-g/liter Bacto yeast extract (Difco), and 5-g/liter NaCl (Sigma, St. Louis, Mo.) supplemented with 4% (wt/vol) glucose (Sigma) and 100-μg/ml kanamycin (Sigma). DH5α does not grow on minimal media due to chromosomal mutations. For nongrowth studies, the cells were harvested from the growth reactor during the mid-growth phase, washed twice with ice-cold phosphate buffer (0.2-g/liter KCl, 5-g/liter NaCl, 2.72-g/liter Na_2HPO_4-$7H_2O$, 0.24-g/liter KH_2PO_4 [Sigma]) and resuspended in phosphate buffer with 2% (wt/vol) glucose as the substrate and 100-μg/ml kanamycin. All cultures were maintained at 30°C, with the pH controlled at 7.0 with 4 M NaOH and with an agitation rate of 300 rpm. Before inoculation, the reactors were sparged with nitrogen gas until the dissolved oxygen concentration reached zero. During cultivation, the reactors were sparged with 0.5 liter of nitrogen gas per min to maintain positive reactor pressure and to maintain anaerobic conditions.

Analytical techniques. PHB content was determined by the method described in reference 38. Glucose concentrations were determined with the Sigma Diagnostics D-glucose kit (Sigma). Ethanol concentrations were determined by adding 0.5 ml of culture supernatant to a gas chromatography vial along with 0.1 ml of internal standard solution (3-g/liter 1-propanol [Sigma]). Samples were run on a Hewlett-Packard (Palo Alto, Calif.) 5890A gas chromatograph with a DB-WAX 30-W capillary column and were analyzed with a flame ionization detector. Acetate levels were analyzed with the R-BIOPHARM (Darmstadt, Germany) acetic acid kit. The by-products lactate, succinate, pyruvate, and 3-hydroxybutyrate were analyzed by the procedure from reference 53 and with a Hewlett-Packard 5890A gas chromatograph and a DB-WAX 30W capillary column with a flame ionization detector. CO_2 measurements were obtained for the nongrowth cultures with a ThermoOnix Prima 8B mass spectrometer (Houston, Tex.). CO_2 production was expressed as the accumulated mass of CO_2 produced per liter of culture. This was done to facilitate comparison with other products.

Metabolic network analysis. The theoretical capabilities of the *E. coli* biochemical network were analyzed by the elementary mode model described previously (12). The reader is referred to this previous work for a detailed description of the metabolic model and for the designation of the individual reactions. Here, we describe only the modifications made to the model to realize the aim of this work. The analysis considered acetate, ethanol, lactate, succinate, and glucose as potential substrates. The designation of the appropriate transport reactions (R90, R91, R94, and R95) was changed from irreversible to reversible. In addition, the designation of reaction R55, which accounts for the interconversion of acetate and acetyl coenzyme A (CoA), was changed to reversible. The additional substrates were included because under anaerobic conditions significant amounts of these compounds are often secreted (1, 6, 15) and would be potentially available for PHB synthesis. To account for PHB synthesis, the following reaction was added to the model:

$$\text{R100: 2 acetyl-CoA + NADH = PHB + 2 CoASH + NAD} \quad (1)$$

This single reaction accounts for the three-gene *R. eutropha* PHB pathway comprising the enzymes β-thiolase, reductase, and synthase. A transhydrogenase activity was assumed to be present. All simulations utilized a macromolecular biomass composition consistent with a 200-min doubling time.

The elementary mode analysis was run with the publicly available program METATOOL version 352_double (34, 42).

The Euclidean distances (44) calculated below only considered native enzymes. Network reactions that account for diffusion-controlled events like the transport of ethanol across the cell membrane were not considered. The Euclidean distance contributions for reversible reactions utilized the absolute value of the flux.

RESULTS

Kinetic studies of PHB production. A series of batch bioreactor experiments were designed to test the feasibility of growth and nongrowth-associated anaerobic PHB synthesis in a recombinant *E. coli* strain. Reactor time profiles for anaerobic growth conditions are shown in Fig. 1 and 2. The control strain PHB(−) also contains a plasmid but is unable to form PHB because it does not express any of the PHB pathway enzymes. The biomass data presented for strain PHB(+) is the residual biomass. Residual biomass is defined as the total dry cell mass minus the mass of PHB.

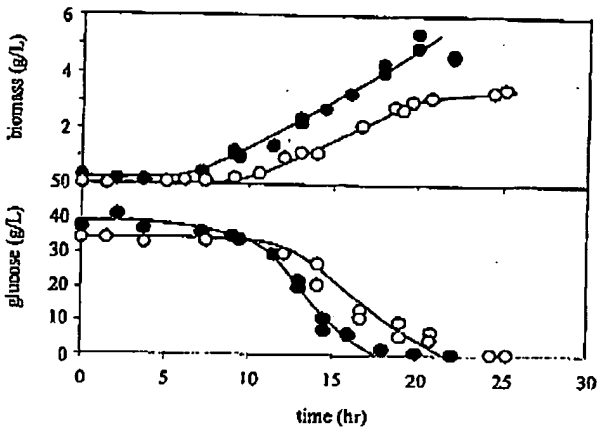

FIG. 1. Residual biomass and glucose time profiles for anaerobic *E. coli* cultures in growth media. The residual biomass is the total biomass minus the mass of PHB. Open circles, strain PHB(+); filled circles, strain PHB(−).

Both recombinant *E. coli* strains grew anaerobically on the rich medium. Strain PHB(−) had a maximum specific growth rate of approximately 0.39 ± 0.03 h^{-1}, while strain PHB(+) expressing the recombinant pathway had a slightly slower maximum specific growth rate of about 0.32 ± 0.04 h^{-1}. The cultures grew exponentially for approximately the first 10 h. However, after this initial culturing period, the biomass only increased at a linear rate. The shift from exponential growth to linear growth is likely due to the accumulation of acetate in the medium or the depletion of a component in the complex medium. The cessation of biomass production coincides closely with the exhaustion of glucose. Strain PHB(−) made approximately 5 g of biomass per liter, while strain PHB(+) made approximately 3 g of residual biomass per liter before the glucose was exhausted.

The recombinant *E. coli* strain PHB(+) was capable of anaerobically synthesizing PHB. The strain accumulated PHB up to approximately 50% of its cell dry weight (cdw). The specific rate of approximately 2.3 ± 0.2 mmol of PHB/g of residual cdw/h is comparable to previously reported rates in recombinant *E. coli* strains growing on rich medium in the presence of oxygen (27, 28). The volumetric PHB production rate and PHB titer are comparable to the majority of the strains tested with batch growth in reference 28; however, improvements are likely possible by screening and selecting an *E. coli* strain best suited for the culturing conditions. High-cell-density strategies, like fed-batch cultivation, would likely further improve the volumetric production rate (27).

Cell growth is not required for anaerobic PHB synthesis. PHB was synthesized anaerobically under nongrowth conditions (see Fig. 3 and 4). The specific PHB content of the

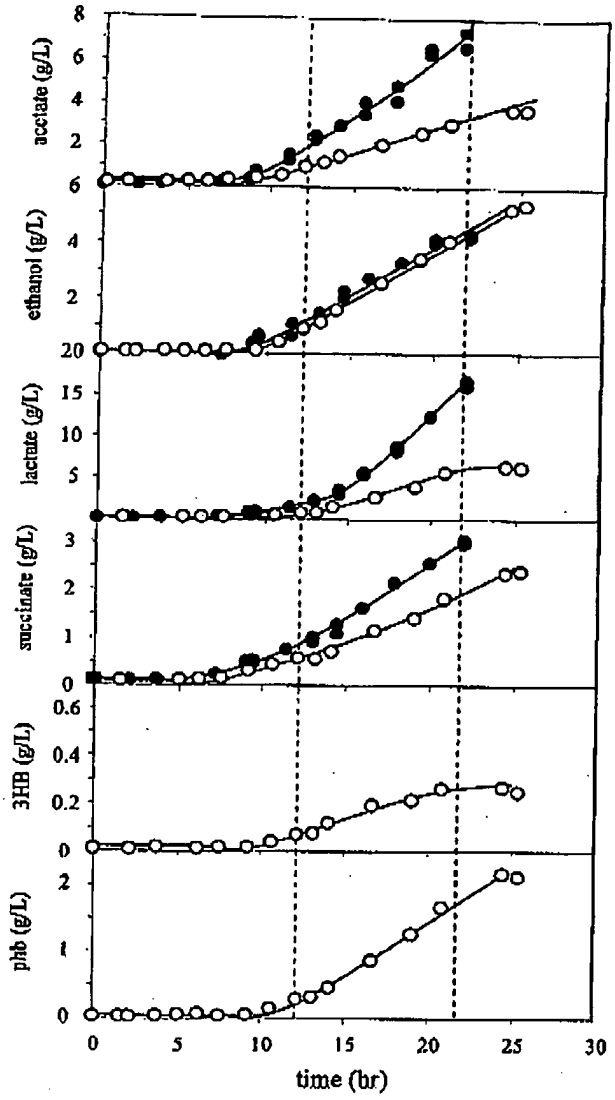

FIG. 2. Product time profiles for anaerobic *E. coli* cultures in growth medium. Open circles, strain PHB(+); filled circles, strain PHB(−). Dotted lines highlight the region used for rate and yield calculations. See text for more detail.

PHB(+) culture increased from approximately 45 to 60% of the cell dry weight. The specific PHB synthesis rate under nongrowth conditions was approximately 0.91 ± 0.2 mmol of PHB/g of residual biomass/h. The ability to produce PHB under nongrowth conditions opens the possibility of novel feeding strategies for producing PHA copolymers and for controlling PHA granule microstructure in *E. coli* (23).

Strain PHB(+) secreted significant amounts of the PHB precursor 3-hydroxybutyric acid during both growth and nongrowth conditions. During the interval between h 12 and 22 for the growth culture, the specific rate of 3-hydroxybutyryl-CoA production is approximately one-seventh the rate of PHB production. 3-Hydroxybutyric acid was secreted under nongrowth conditions at a rate of about 1.1 ± 0.2 mmol/g of residual biomass/h. The PHB monomer, 3-hydroxybutyryl-CoA, was likely cleaved into 3-hydroxybutyric acid and free CoA by a native *E. coli* thioesterase.

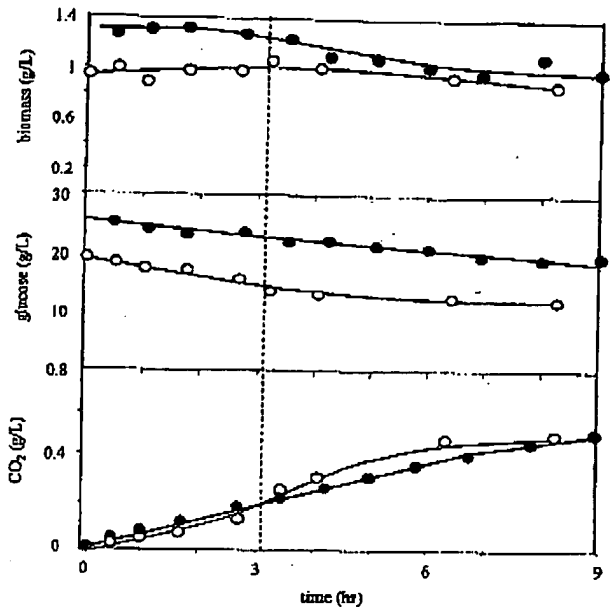

FIG. 3. Residual biomass, glucose, and CO_2 time profiles for anaerobic *E. coli* cultures in nongrowth medium. Open circles, strain PHB(+); filled circles, strain PHB(−).

PHB synthesis had a significant effect on the production of by-products for both growth and nongrowth conditions. Strain PHB(−) accumulated significantly more acetate than strain PHB(+), while strain PHB(+) accumulated more ethanol than strain PHB(−). Under growth conditions, strain PHB(−) made significantly more lactate and succinate than strain PHB(+). These trends are likely due to the high levels of accumulated acetate.

By-product yields. The metabolic differences between the two strains were quantified by examining the by-product glucose yields. The product yield, Y, based on glucose is defined as

$$Y_{\text{glucose}}^{\text{product}} = \frac{r_{\text{product}}}{-r_{\text{glucose}}} = \frac{[\text{product produced}]}{[\text{glucose consumed}]} \qquad (2)$$

where r is the rate of either by-product secretion or glucose consumption. Since the volumetric rates $\left(\frac{d[\text{product}]}{dt}\right)$ for the products were nearly linear between h 12 and 22 for the growth cultures and between h 0.5 and 3 for the nongrowth conditions, the culture yields were nearly constant during these intervals. More than 67% of all PHB synthesis occurred during these intervals. The following analysis is limited to these culturing phases.

The reactor yields for both growth and nongrowth conditions are plotted as the ratio of specific product secretion rates to the specific glucose uptake rate in Fig. 5. The rates calcu-

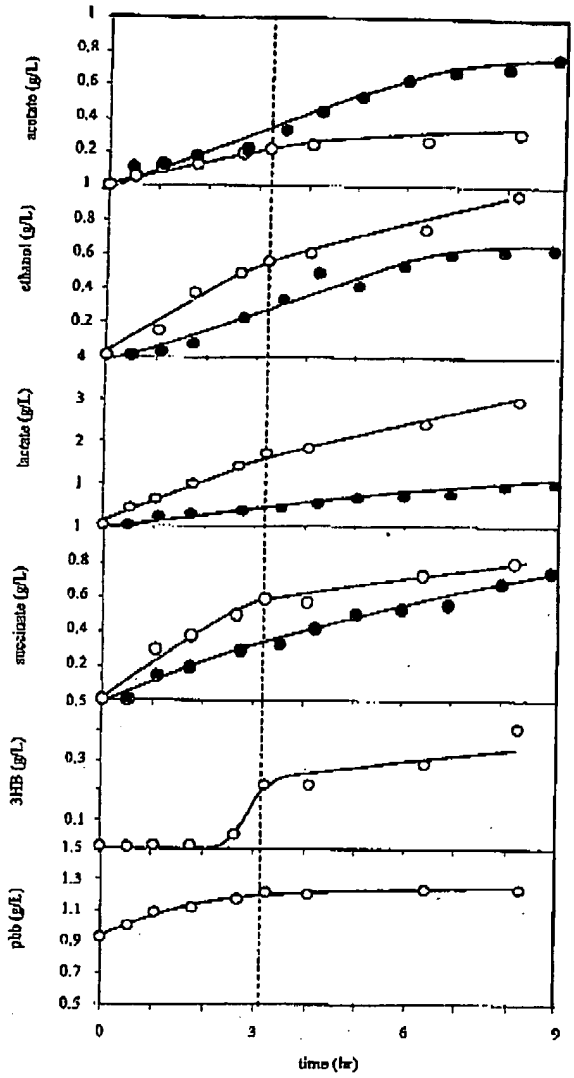

FIG. 4. Product time profiles for anaerobic *E. coli* cultures in nongrowth medium. Open circles, strain PHB(+); filled circles, strain PHB(−). Dotted lines highlight the region used for rate and yield calculations. See text for more details.

lated from the experimental cultures are shown as data points, while the slopes of the lines are equivalent to the product yield based on equation 2. The numerical value for each yield, in terms of moles of carbon (C moles) of product per C mole of glucose, is given in each plot.

The two strains utilized different ratios of metabolic intermediates as reducing equivalent sinks. Strain PHB(−) had a larger acetate, lactate, and succinate yield than PHB(+) under growth conditions, while strain PHB(+) had a higher ethanol yield under growth conditions. The growth-associated PHB yield was approximately equal to the nongrowth-associated PHB yield. The 3-hydroxybutyric acid yield was significantly higher under nongrowth conditions. This is likely due to the high intracellular PHB content, which may have limited additional polymerization of 3-hydroxybutyryl-CoA and favored the secretion of the free acid.

Interestingly, Fig. 5 shows that even with a higher extracellular concentration of potentially toxic acetate, strain PHB(−) during growth and nongrowth conditions had a higher acetate yield on glucose.

Only trace amounts of pyruvate were detected. H_2 and formate concentrations were not determined. CO_2 concentrations were not measured for the growth conditions.

Based on the carbon yields in Fig. 5 and the CO_2 data, approximately 68 and 87% of the utilized carbon during nongrowth conditions was recovered for the PHB(−) and PHB(+) cultures, respectively. If it is assumed that one formate molecule is formed via pyruvate formate-lyase during the production of each acetate and each ethanol molecule and that each 3-hydroxybutyrate and each PHB molecule results in the production of two formate molecules, the carbon recoveries based on Fig. 5 improve to approximately 80 and 104% for the PHB(−) and PHB(+) cultures, respectively. These formate-corrected yields were used to determine a redox balance, which is expressed here as the ratio of an electron balance on the oxidized glucose to the produced fermentation products. The PHB(−) and PHB(+) cultures had ratios of 1.28 and 0.97, respectively. The carbon and redox shortcomings in the PHB(−) culture are believed to be the result of analytical error. A carbon and redox balance was not attempted for the growth conditions since the complex medium contained approximately 26 g of undefined components per liter (see Materials and Methods). Therefore, a considerable amount of the carbon is unaccounted for in the recovered by-products. However, even without more detailed data, it is apparent that anaerobic production of PHB is possible and that it has a significant effect on cellular metabolism.

DISCUSSION

The cellular redox state during anaerobic cultivation likely favors PHB production. A high NADPH/NADP ratio has been shown to stimulate PHB production (17). This is probably due to the regulation pattern of the β-ketothiolase enzyme and an increased flux through the reductase-catalyzed step in the PHB pathway which consumes reduction equivalents. This finding highlights the role of PHB not only as a carbon reserve compound but also as a redox sink. de Graf et al. (18) and Alexeeva et al. (2) studied the redox (NADH/NAD) ratios in *E. coli* under different levels of oxygen limitation, including anaerobic conditions. Under anaerobic growth conditions, the ratio of NADH to NAD is estimated to be more than 10 times higher than that under fully aerobic growth conditions. *E. coli* possesses at least two transhydrogenase systems (7, 8), so a high NADH/NAD ratio would likely favor a high NADPH/NADP ratio, which could stimulate PHB production.

The presented work is an experimental study of anaerobic PHB production in *E. coli*. In addition, we have attempted to interpret the observed differences in by-product yields in terms of network fluxes. A biochemical pathway analysis technique known as elementary mode analysis (40–43) was utilized to provide a rational basis for interpreting the results from a systems biology basis. The analysis method was used to inves-

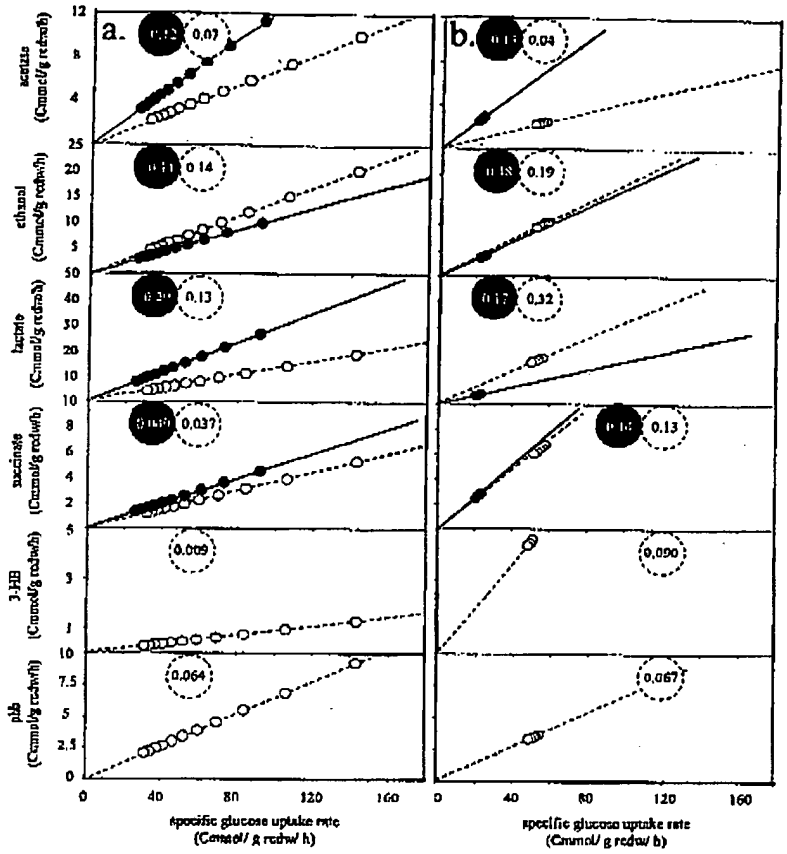

FIG. 5. Bioreactor yields for cultures under (a) growth conditions and (b) nongrowth conditions expressed as the ratio of the specific product secretion rate to the specific glucose uptake rate. All rates are expressed as C millimoles per gram of residual cell dry weight (redw) per hour. Filled circles and solid lines represent data from strain PHB(−), while open circles and dashed lines represent data from strain PHB(+). Numerical values for the yields are shown in the upper left of each panel. The filled circle is the product yield in terms of C millimoles per C millimole of glucose for strain PHB(−), while the open, dashed circle is the carbon yield for strain PHB(+).

tigate three questions related to the experimental results and two theoretical questions related to network properties as follows.

(i) **Anaerobic PHB production.** Elementary mode analysis was first used to determine whether the network model could predict the feasibility of anaerobic PHB production. The analysis identified a total of 202 anaerobic PHB-producing pathways. Ninety-eight modes made PHB without coproducing biomass. These results are summarized in Table 1. Product carbon yield is used as a measure of pathway efficiency and is defined here as the ratio of the C moles in the product to the number of C moles in the substrate(s). The most efficient anaerobic PHB synthesis mode has a carbon yield of 0.8 (Fig. 6). This mode represents the upper limit of network performance. However, the mode does not account for critical processes like maintenance energy generation, which would lower the observed product yield.

TABLE 1. Summary of elementary mode analysis of a recombinant *E. coli* biochemical network

Mode	No. of modes
Total aerobic and anaerobic	10,269
Total anaerobic	836
Total biomass (anaerobic)	461
Biomass (anaerobic, no PHB)	357
Total PHB (anaerobic)	202
PHB (anaerobic, no biomass)	98

(ii) PHB synthesis lowers acetate yields and increases ethanol yields. To explain these observations in terms of network fluxes, a method capable of estimating the minimum network perturbation was applied to the possible anaerobic, nongrowth PHB pathways. This method, known as minimization of metabolic adjustment, predicts the behavior of a perturbed system by assuming a network will minimize the readjustment of native, optimal fluxes (44). The theory was developed on an *E. coli* knockout mutant and is examined here on a recombinant host expressing a foreign pathway.

The set of all 98 possible anaerobic PHB pathways was analyzed for their Euclidean distance from the most efficient,

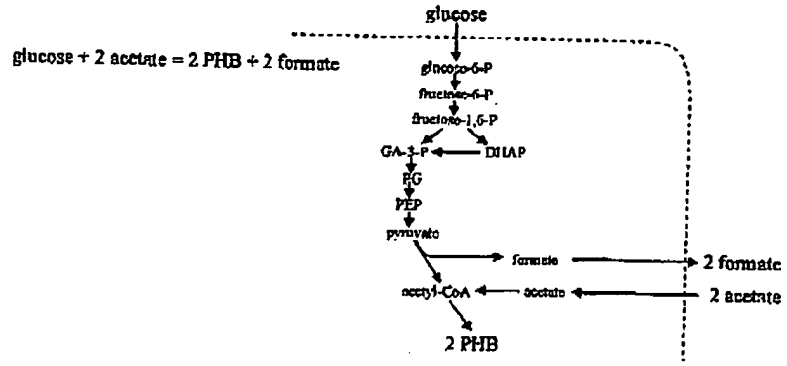

FIG. 6. Most efficient anaerobic PHB-synthesizing pathway for the recombinant *E. coli* biochemical network. PHB carbon yield is 0.8.

native ATP-producing pathway, which was identified as M_4^{ATP} in reference 12. This pathway was examined because under nongrowth conditions it is heavily utilized to satisfy maintenance energy requirements. The Euclidean distance, D_j, between a given anaerobic PHB pathway, M_j^{PHB}, and the optimal anaerobic energy pathway, M_4^{ATP}, is defined as follows

$$D_j(M_j^{PHB}, M_4^{ATP}) = \sqrt{\sum_{i=1}^{R}(m_{i,j}^{PHB} - m_{i,4}^{ATP})^2} \quad j = 1,98 \quad (3)$$

where R is the number of reactions in the pathway model and m is the flux through reaction i of elementary mode j. All pathways were normalized to reflect a flux of 1 glucose mol prior to comparison. Smaller Euclidean distances represent a smaller perturbation of native fluxes. Smaller Euclidean distances could therefore be a characteristic of a recombinant pathway which is likely to be utilized. The calculated distances varied between 1.11 and 30.25 mol/mol of glucose.

The overall stoichiometries of the six pathways with the shortest Euclidean distances to the energy pathway M_4^{ATP} are shown in Table 2. These pathways show two trends consistent with experimental results. Four of the pathways (modes 2 to 5) consume acetate along with glucose to make PHB. These pathways suggest that PHB production would lower the observed acetate yield. Four of the pathways (modes 1, 3, 4, and 6) coproduce ethanol along with PHB. These pathways suggest that PHB production would increase the observed ethanol production. During both growth and nongrowth experiments, strain PHB(+) accumulated less acetate and accumulated more ethanol than strain PHB(−) (Fig. 2 and 4). Thus, the concept of the smallest Euclidean distance appears to be consistent with the observed behavior.

As discussed in reference 13, maintenance energy pathways are also active during biomass production. It is therefore reasonable to assume that the results of this analysis apply as well to growth conditions.

(iii) Production of lactate and succinate. The most efficient pathway for anaerobic growth and energy production in terms of glucose carbon yield (0.5 ATP per C mol of glucose) produces only acetate, ethanol, and formate as by-products (pathway M_4^{ATP}; see Table 2) (12). However, there is often substantial accumulation of lactate and succinate during anaerobic culturing (Fig. 2 and 4). The next most efficient pathways have an ATP/C mol of glucose yield of 0.33 and produce either lactate or a combination of succinate, acetate, and formate (pathways not shown). Lactate production in *E. coli* has been linked to cultivation at acidic pHs because, as compared to acetate, lactate is less toxic (19, 25). Lactate is also actively transported out of the cell, which may permit faster NAD^+ regeneration fluxes than diffusion of acetate and ethanol through the cell boundaries (5, 30). The production of succinate, which is also actively transported out of the cell, likely represents a strategy similar to the lactate-producing mode.

(iv) Optimal pathways for coproducing PHB and another valuable or less toxic compound like ethanol, lactate, succinate, or H_2. From a practical standpoint, it could be advanta-

TABLE 2. Anaerobic PHB-producing pathways that represent the shortest Euclidean distance from the optimal anaerobic energy-producing pathway[a]

Pathway	Euclidean distance (mol/mol of glucose)
M_4^{ATP} glucose = 3 ATP + acetate + ethanol + formate	0
1. 3 glucose = 6 ATP + 2 ethanol + 6 formate + 2 PHB	1.11
2. glucose + 2 acetate = 2 formate + 2 PHB	2.0
3. 3 glucose + 5 acetate = 4 ethanol + 5 formate + 3 CO_2 + 3 PHB	4.22
4. 3 glucose + 3 acetate = ethanol + 6 formate + 4 PHB	4.44
5. 3 glucose + 3 acetate + CO_2 = succinate + 5 formate + 4 PHB	5.0
6. 3 glucose = 3 ATP + 2 ethanol + 6 formate + 2 PHB	5.11

[a] The modes are listed in ascending order based on the Euclidean distance. The most efficient anaerobic energy-generating mode is listed at the top for comparison. See text for more details.

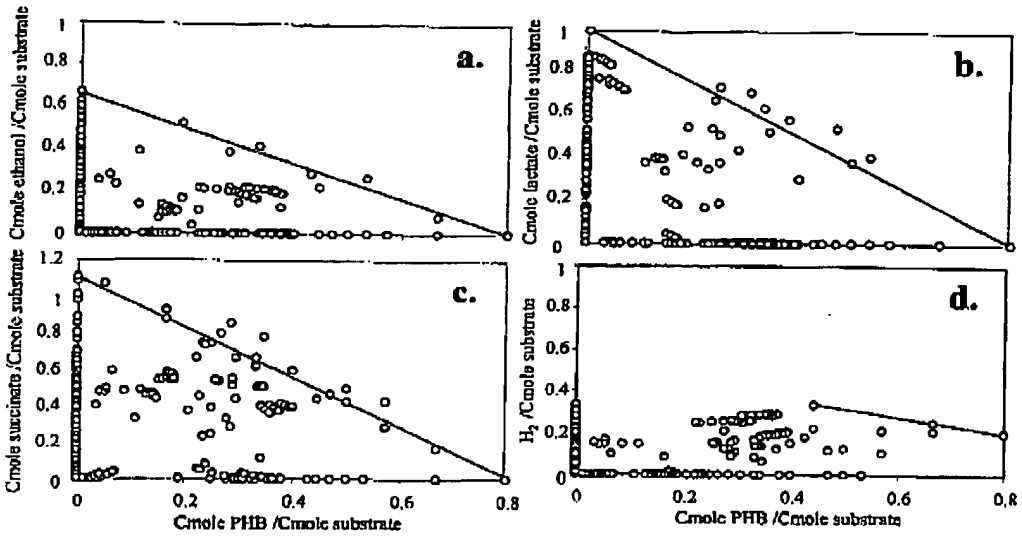

FIG. 7. Relationship between product carbon yields for multiple products. Carbon yield was defined as the ratio of C moles in product to the number of C moles in the substrate(s). The H_2 yield represents the moles of H_2 produced per C mole of substrate(s). a, Ethanol and PHB; b, lactate and PHB; c, succinate and PHB; and d, H_2 and PHB. The line represents the yield performance of linear combinations of the two highest-yielding single-product pathways. Modes above this line represent a higher conversion of substrate carbon into coproducts.

geous to direct fluxes away from toxic compounds like acetate toward either less toxic or more valuable by-products. Figure 7 examines the theoretical capabilities of the *E. coli* biochemical network to anaerobically coproduce ethanol (Fig. 7a), succinate (Fig. 7b), lactate (Fig. 7c), and H_2 (Fig. 7d) along with PHB. Some of the carbon yields for succinate are greater than 1 due to the assimilation of CO_2 via PEP carboxylase. H_2 production is assumed to occur from formate via formate hydrogen-lyase.

A straight line is used to connect the highest-yielding anaerobic PHB mode with the highest-yielding mode for the other considered by-products. This line represents the relationship between the yields of the two products during coproduction if only linear combinations of the two end point modes are considered. When coproduction of two products is desired, the plot reveals that linear combinations of the two highest-yielding, single-product modes is not always the most efficient use of the metabolic network. Modes that lie above the line represent strategies that are more efficient in terms of total substrate carbon recovery than those using linear combinations of the most efficient modes for each single product. While Fig. 7 represents the substrate carbon yield, other considerations like the commercial value of each product can also be explored.

(v) Comparison of PHB production in *E. coli* and *Saccharomyces cerevisiae*. Numerous studies have examined PHA expression in different recombinant systems with the intention of taking advantage of each unique biochemical network (for example, see references 20, 26, 35, 36, 47). A comparison of the most efficient anaerobic PHB-producing modes in *E. coli* and *S. cerevisiae* reveals very different theoretical network capabilities. The most efficient *E. coli* pathway has a theoretical PHB carbon yield of 0.8 (Fig. 6), while the most efficient *S. cerevisiae* pathway has a theoretical PHB carbon yield of only 0.24 (11). The topological features of both biochemical networks are similar, but the absence of two key enzymatic activities in *S. cerevisiae* lowers its PHB carbon yield. Unlike *E. coli*, *S. cerevisiae* does not possess a transhydrogenase system (9, 21) nor does it possess a pyruvate formate-lyase activity. The lack of these enzymatic activities lowers the anaerobic PHB yield.

The anaerobic synthesis of PHB was demonstrated in a recombinant *E. coli* strain under both growth and nongrowth conditions. The recombinant pathway had a significant effect on by-product secretion patterns. The system represents a potentially interesting strategy for PHA synthesis and provides insight into the *E. coli* central metabolism.

ACKNOWLEDGMENTS

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REFERENCES

1. Alam, K. Y., and D. P. Clark. 1989. Anaerobic fermentation balance of *Escherichia coli* as observed by in vivo nuclear magnetic resonance spectroscopy. J. Bacteriol. 171:6213–6217.
2. Alexeeva, S., B. de Kort, G. Sawers, K. J. Hellingwerf, and M. J. Teixeira de Mattos. 2000. Effects of limited aeration and of the ArcAB system on intermediary pyruvate catabolism in *Escherichia coli*. J. Bacteriol. 182:4934–4940.
3. Amos, D. A., and M. J. McInerney. 1989. Poly-β-hydroxyalkanoate in *Syntrophomonas wolfei*. Arch. Microbiol. 152:172–177.
4. Amos, D. A., and M. J. McInerney. 1991. Composition of poly-β-hydroxyalkanoates from *Syntrophomonas wolfei* grown on unsaturated fatty acid substrates. Arch. Microbiol. 155:103–106.
5. Axe, D. D., and J. E. Bailey. 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. Biotechnol. Bioeng. 47:8–19.
6. Blackwood, A. C., A. C. Nelsh, and G. A. Ledingham. 1956. Dissimilation of glucose at controlled pH values by pigmented and non-pigmented strains of *Escherichia coli*. J. Bacteriol. 72:497–499.
7. Boonstra, B., C. E. French, I. Wainwright, and N. C. Bruce. 1999. The *udhA*

gene of *Escherichia coli* encodes a soluble pyridine nucleotide transhydrogenase. J. Bacteriol. 181:1030–1034.
8. **Bragg, P. D., P. L. Davies, and C. Hou.** 1972. Function of energy-dependent transhydrogenase in *Escherichia coli*. Biochem. Biophys. Res. Commun. 47: 1248–1255.
9. **Bruinenberg, P. M., R. Jonker, J. P. van Dijken, and A. Scheffers.** 1985. Utilization of formate as an additional energy source by glucose-limited chemostat cultures of *Candida utilis* CBS 621 and *Saccharomyces cerevisiae* CBS 8066: evidence for absence of transhydrogenase activity in yeasts. Arch. Microbiol. 142:302–306.
10. **Cameron, D. C., M. L. Altaras, M. L. Hoffman, and A. J. Shaw.** 1998. Metabolic engineering of propanediol pathways. Biotechnol. Prog. 14:116–125.
11. **Carlson, R., D. Fell, and F. Srienc.** 2002. Metabolic pathway analysis of a recombinant yeast for rational strain development. Biotechnol. Bioeng. 79: 121–134.
12. **Carlson, R., and F. Srienc.** 2004. Fundamental *Escherichia coli* biochemical pathways for biomass and energy production: identification of reactions. Biotechnol. Bioeng. 85:1–19.
13. **Carlson, R., and F. Srienc.** 2004. Fundamental *Escherichia coli* biochemical pathways for biomass and energy production: construction of overall flux state. Biotechnol. Bioeng. 86:149–162.
14. **Chang, D.-E., H.-C. Jung, J.-S. Rhee, and J.-G. Pan.** 1999. Homofermentative production of D- or L-lactate in metabolically engineered *Escherichia coli* RR1. Appl. Environ. Microbiol. 65:1384–1389.
15. **Chesbro, W., T. Evans, and R. Eifert.** 1979. Very slow growth of *Escherichia coli*. J. Bacteriol. 139:625–638.
16. **Choi, J.-I., and S. Y. Lee.** 1999. High-level production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch culture of recombinant *Escherichia coli*. Appl. Environ. Microbiol. 65:4363–4368.
17. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. 10:135–266.
18. **de Graf, M. R., S. Alexeeva, J. L. Snoep, and M. J. Teixeira de Mattos.** 1999. The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. J. Bacteriol. 181:2351–2357.
19. **Diez-Gonzalez, F., and J. B. Russell.** 1997. Effects of carbonylcyanide-m-chlorophenylhydrazone (CCCP) and acetate on *Escherichia coli* O157:H7 and K-12: uncoupling versus anion accumulation. FEMS Microbiol. Lett. 151:71–76.
20. **Hahn, J. J., A. Eschenlauer, M. Narrol, D. Somers, and F. Srienc.** 1997. Growth kinetics, nutrient uptake, and expression of the *Alcaligenes eutrophus* poly(beta-hydroxybutyrate) synthesis pathway in transgenic maize cell suspension cultures. Biotechnol. Prog. 13:347–351.
21. **Hoek, J. B., and J. Rydström.** 1988. Physiological roles of nicotinamide nucleotide transhydrogenase. Biochem. J. 254:1–10.
22. **Ingram, L. O., P. F. Gomez, X. Lai, Monirruzzaman, B. E. Wood, L. P. Yomano, and S. W. York.** 1998. Metabolic engineering of bacteria of ethanol production. Biotechnol. Bioeng. 58:204–214.
23. **Kelley, A. S., and F. Srienc.** 1999. Production of two phase polyhydroxyalkanoic acid granules in *Ralstonia eutropha*. I. J. Biol. Macromol. 25:61–67.
24. **Kidwell, J., H. E. Valentin, and D. Dennis.** 1995. Regulated expression of the *Alcaligenes eutrophus pha* biosynthesis genes in *Escherichia coli*. Appl. Environ. Microbiol. 61:1391–1398.
25. **Kirkpatrick, C., L. M. Maurer, N. E. Oyelakin, Y. N. Yoncheva, R. Maurer, and J. L. Slonczewski.** 2001. Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*. J. Bacteriol. 183:6466–6477.
26. **Leaf, T., M. Peterson, S. Stoup, D. Somers, and F. Srienc.** 1996. *Saccharomyces cerevisiae* expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiology 142:1169–1180.
27. **Lee, S. Y., and H. N. Chang.** 1995. Production of poly(3-hydroxybutyric acid) by recombinant *Escherichia coli* strains: genetic and fermentation studies. Can. J. Microbiol. 41:207–215.
28. **Lee, S. Y., K. M. Lee, H. N. Chang, and A. Steinbüchel.** 1994. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric acid) and morphological changes. Biotechnol. Bioeng. 44: 1337–1347.
29. **Lino, J. C., S.-Y. Hou, and Y.-P. Chao.** 1996. Pathway analysis, engineering and physiological considerations for redirecting central metabolism. Biotechnol. Bioeng. 52:129–140.
30. **Matin, A., and W. N. Konings.** 1973. Transport of lactate and succinate by membrane vesicles of *Escherichia coli*, *Bacillus subtilis*, and a *Pseudomonas* species. Eur. J. Biochem. 34:58–67.
31. **Mino, T., M. C. M. Van Loosdrecht, and J. J. Heijnen.** 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. Water Res. 32:3193–3207.
32. **Peoples, O., and A. Sinskey.** 1989. Poly-beta-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding beta-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. 264: 15293–15297.
33. **Peoples, O., and A. Sinskey.** 1989b. Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. 264:15298–15303.
34. **Pfeiffer, T., I. Sánchez-Valdenebro, J. C. Nuño, F. Montero, and S. Schuster.** 1999. METATOOL: for studying metabolic networks. Bioinformatics 15: 251–257.
35. **Poirier, Y., N. Erard, and J. MacDonald-Comber Petétot.** 2002. Synthesis of polyhydroxyalkanoate in the peroxisome of *Pichia pastoris*. FEMS Microbiol. Lett. 207:97–102.
36. **Poirier, Y., N. Erard, and J. Macdonald-Comber Petétot.** 2001. Synthesis of polyhydroxyalkanoate in the peroxisomes of *Saccharomyces cerevisiae* by using intermediates of fatty acid β-oxidation. Appl. Environ. Microbiol. 67:5254–5260.
37. **Randall, A. A., and Y. H. Liu.** 2002. Polyhydroxyalkanoates form potentially a key aspect of aerobic phosphorous uptake in enhanced biological phosphorous removal. Water Res. 36:3473–3478.
38. **Riis, V., and W. Mai.** 1988. Gas chromatographic determination of poly-β-hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. J. Chromatogr. 445:285–289.
39. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. 170:5837–5847.
40. **Schuster, S., T. Dandekar, and D. A. Fell.** 1999. Detection of elementary flux modes in biochemical networks: a promising tool for pathway analysis and metabolic engineering. Trends Biotechnol. 17:53–60.
41. **Schuster, S., D. A. Fell, and T. Dandekar.** 2000. A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. Nat. Biotechnol. 18:326–332.
42. **Schuster, S., C. Hilgetag, and D. A. Fell.** 1994. Detecting elementary modes of functioning in metabolic networks. Mod. Trends BioThermoKinetics 3:103–105.
43. **Schuster, S., C. Hilgetag, J. H. Woods, and D. A. Fell.** 2002. Reaction routes in biochemical reaction systems: algebraic properties, validated calculation procedure and example from nucleotide metabolism. J. Math. Biol. 45:153–181.
44. **Segre, D., D. Vitkup, and G. M. Church.** 2002. Analysis of optimality in natural and perturbed metabolic networks. Proc. Natl. Acad. Sci. USA 99: 15112–15117.
45. **Senior, P. J., G. A. Beech, G. A. Ritchie, and E. A. Dawes.** 1972. The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. Biochem. J. 128:1193–1201.
46. **Seviour, R. J., T. Mino, and M. Onuki.** 2003. The microbiology of biological phosphorous removal in activated sludge systems. FEMS Microbiol. Rev. 27:99–127.
47. **Slater, S., W. Volpe, and D. Dennis.** 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. 170:4431–4436.
48. **Steinbüchel, A.** 1991. Polyhydroxyalkanoic acids, p. 123–214. *In* D. Byrom (ed.), Biomaterials: novel materials from biological sources. Stockton Press, New York, N.Y.
49. **Stelling, J., S. Klamt, K. Bettenbrock, S. Schuster, and E. D. Gilles.** 2002. Metabolic network structure determines key aspects of functionality and regulation. Nature 420:190–193.
50. **Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton.** 2002. Biofilms as complex differentiated communities. Annu. Rev. Microbiol. 56:187–209.
51. **Sudesh, K., H. Abe, and Y. Doi.** 2000. Synthesis, structure and properties of polyhydroxyalkanoates:. biological polyesters. Prog. Polym. Sci. 25: 1503–1555.
52. **Van Wegen, R. J., S. Y. Lee, and A. P. J. Middelberg.** 2001. Metabolic and kinetic analysis of poly(3-hydroxybutyrate) production by recombinant *Escherichia coli*. Biotechnol. Bioeng. 74:70–80.
53. **Vollbrecht, D., M. A. El Nawawy, and H. G. Schlegel.** 1978. Excretion of metabolites by hydrogen bacteria. I. Autotrophic and heterotrophic fermentations. Eur. J. Appl. Microbiol. 6:145–155.
54. **Wang, F., and S. Y. Lee.** 1997. Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. Appl. Environ. Microbiol. 63:4765–4769.

RELATED PROCEEDINGS APPENDIX

None.